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Alteration of lipid membrane rigidity by cholesterol and its metabolic precursors

Horia I. Petrache, Daniel Harries, V. Adrian Parsegian*

Laboratory of Physical and Structural Biology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892. E-mail: petrachh@mail.nih.gov

Summary: Caused by biosynthesis defects, cholesterol deficiency can lead to developmental disorders and malformations, with possible implication of lipid membrane properties. We show that modification of sterol chemical structure alters membrane physical properties significantly. By X-ray diffraction and osmotic stress, we measure changes in the bending rigidity of bilayers containing either cholesterol or one of its metabolic precursors. Membrane elasticity differs dramatically between slightly different sterols and varies in the sequence lanosterol < 7-dehydrocholesterol < lathosterol < cholesterol. We interpret the results in terms of sterol location within lipid structures and modification of lateral stress, a structural feature relevant to interactions within biological membranes. We find that cholesterol is most efficient in enhancing membrane rigidity, a possible clue to why depletion or replacement with other sterols can affect cellular structures.

Keywords: bending rigidity, cholesterol biosynthesis, intrinsic curvature, stress profile, X-ray

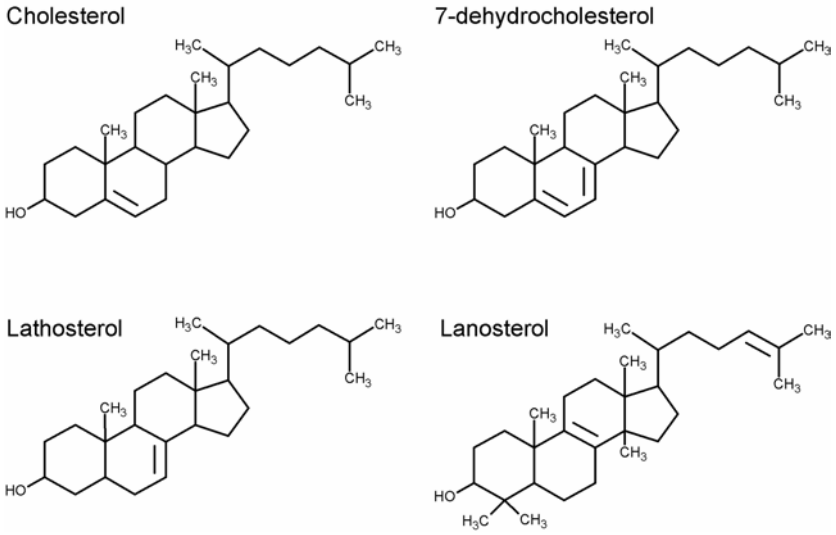
Lipid bilayers, cholesterol, and cellular function

To a physical chemist, cellular membranes are molecular alloys: their material properties subtly depend on composition, temperature, and other environmental variables. Do all relevant biomembrane functions rely on pure biochemistry, or do they also depend on physical properties of the molecular assembly?

As a rule of thumb, the larger the fraction of a given membrane component, the more likely it is to modulate a material property. This rule holds for cholesterol. Present in plasma membrane of all mammalian cells at mole fractions as high as 30-50% of total lipid, cholesterol likely acts through non-specific physical properties as well as specific interactions.[1,2]

To a cell biologist, cholesterol biosynthesis[3] is a tightly regulated, multistep chemical process via numerous precursors including lanosterol, lathosterol, desmosterol and 7-dehydrocholesterol. As is widely recognized, excess cholesterol leads to atherosclerosis, cardiovascular diseases, and stroke. Deficiency is also dangerous, leading to serious congenital anomalies and mental retardation in newborns.[4,5] Despite numerous studies of cholesterol and its precursors, detailed understanding of sterol-lipid interactions in relation with membrane architecture is still lacking.

Structural differences between cholesterol and its precursors include the number and position of double bonds and additional methyl groups.



Can bilayer material properties conferred by cholesterol differ significantly from its precursors? To what extent can additional double bonds and methyl groups modify sterol-lipid interactions? To address these questions, we analyze interbilayer interactions and curvatures of hexagonal phases. We follow a simple procedure to obtain indirect information on bending rigidities, and find that cholesterol makes significantly more rigid bilayers than other sterols in its biosynthesis pathway. These results can be of significance to understanding the molecular mechanisms responsible for manifestations of cholesterol-related disorders.

The balance of forces within lipid bilayers

From the heterogenous molecular structure of lipids themselves and from the tumultuous water/lipid headgroup region[6], aggregated lipids are subjected to a non-uniform

distribution of forces (lateral stress).[7-10] As depicted in Figure 1, this inhomogenous distribution of forces along the bilayer normal generates bending moments.[11] Were they not paired into bilayers, individual monolayers would generally tend to deviate from planar geometry. A lipid bilayer composed of frustrated monolayers lives under stress. It turns out that the balance of forces within bilayers can easily be offset by additives such as sterols. [1,7]

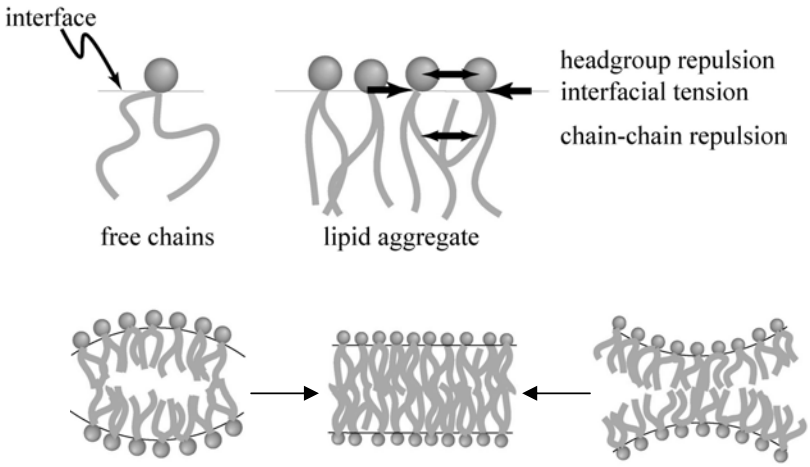


Figure 1. Schematics of the inhomogeneous distribution of lateral forces within lipid bilayers. Monolayer bending tendency (moments) lead to packing frustration.

Within this framework, we can think of sterol effects as changes in monolayer bending moments. This change is measured by the monolayer bending rigidity, K_C^{mono}, and the monolayer intrinsic curvature, $1/R_0$. The intrinsic curvature is the value at which the monolayer free energy is minimum in the absence of other terms, e.g. monolayer coupling. In a harmonic approximation, the free energy per area needed to bend a monolayer away from R_0 is[12],

$$F_{elastic}^{m}(R) \approx \frac{1}{2} K_C^{mono} \left(\frac{1}{R} - \frac{1}{R_0} \right)^2 . \qquad (1)$$

Roughly speaking, K_C^{mono} represents the energy needed to flatten a spontaneously curved monolayer of area $2R_0^2$. To calculate the free energy of bending a *bilayer*, we sum up the bending energies of paired monolayers, taking into account their opposite direction of bending (competing springs). In the thin film approximation, the bilayer bending rigity has the form

$$K_C \cong 2K_C^{mono}[1 + \vartheta(D_B / R_0)], \quad (2)$$

where $\vartheta(D_B / R_0)$ indicates a correction on the order of D_B / R_0. The bilayer bending rigidity is larger than twice the rigidity of a monolayer, a stiffening effect due to the finite thickness D_B. For many bilayer-forming phospholipids, K_C is on the order of $\sim 10^{-12}$ erg $\approx 23\ k_B T$[13].

The method of choice here to probe this property is the measurement of the response of lipid aggregates to osmotic stress.[14-16] Multilamellar lipid bilayers are hydrated in the presence of osmolytes, e.g. high-molecular weight polyethyleneglycol (PEG), and interbilayer separation is measured, using X-rays, as a function of the applied osmotic stress set by the PEG/water weight ratio. These measurements produce force-distance curves analogous to pressure-volume curves of fluids, as will be shown below.

X-ray images of lipid phases

To study sterol effects on bending moments and intrinsic curvatures, we use lipids which, depending on their intrinsic curvature values, self-assemble into either lamellar (L_α) or inverted hexagonal (H_{II}) phases at physiological temperatures. Lipids with saturated acyl chains and phosphatidylcholine (PC) headgroups form bilayer phases. Conversely, lipids with unsaturated chains and phosphatidylethanolamine headgroups (PE, a demethylated PC) form inverse hexagonal phases. We have chosen the 14-carbon disaturated dimyristoylphosphatidylcholine (DMPC) and the monounsaturated 18-carbon dioleoylphosphatidylethanolamine (DOPE) as representative of each class. The geometry of lipid aggregates, and corresponding X-ray pictures are shown in Figure 2. Rings rather than points of scattering are obtained due to random, "powder" orientations of lipid suspensions in the X-ray beam. The lattice (repeat) spacings are determined from the position of these rings. For lamellar structures, the rings are equally spaced and index simply as 1,1/2, 1/3, …, while for the H_{II} phase the indexing is 1,1/√3, 1/2, 1/√7, ...[8,12]

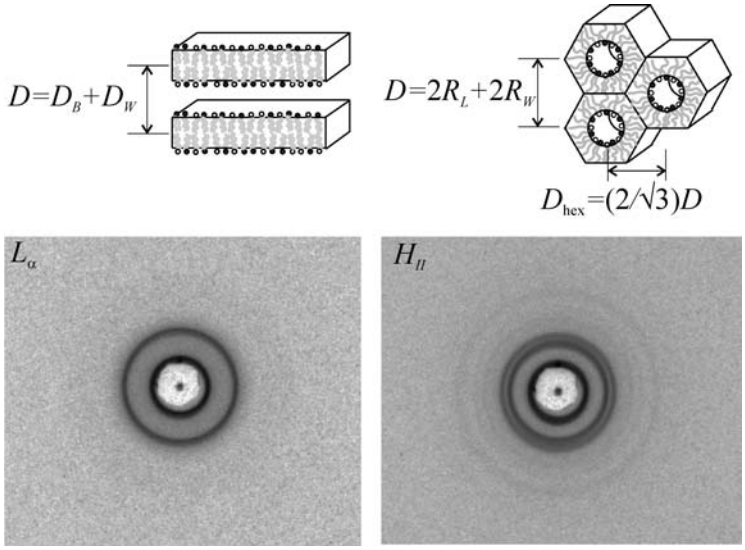


Figure 2. X-ray scattering from multilamellar (L_α) DMPC and inverted hexagonal (H_{II}) DOPE, both fully hydrated at 35°C. Scattering rings of DMPC are equally spaced and reflect the regular spacing between stacked bilayers. The X-ray rings of DOPE index as 1,1/√3, 1/2, 1/√7,… and reflect the honeycomb-like positioning of water cylinders in the H_{II} phase.

DOPE has a negative spontaneous curvature and forms an inverted hexagonal phase when fully hydrated. DMPC, with a much smaller spontaneous curvature, forms a lamellar phase. The structures depicted in Figure 2 typically extend to 10^2-10^3 bilayers (DMPC) or cylinders (DOPE) in a given scattering domain, as determined from the sharpness of the scattering peaks. For fully hydrated DMPC in water at 35°C, the interlamellar repeat spacing is D = 63 Å, which we decompose into a bilayer thickness D_B = 44 Å [16], and a water spacing of D_W = 19 Å (see drawing in Figure 2). For DOPE, with a hexagonal lattice spacing of D_{hex} = 64 Å, D = 74 Å, with $2R_L$ = 36 Å and $2R_W$ = 38 Å[17]. The water content, measured by D_W and R_W, is reduced under osmotic stress.

The highly curved hexagonal phases and the way in which they respond to osmotic stress (dehydration) have been directly related to bending energies.[12,17-19] For the most biologically relevant lamellar geometry, however, in which curvature is not explicit, we obtain information on bilayer elasticity from measurements of membrane shape fluctuations.[16,20-23]

Bilayer interactions

For neutral fluid membranes, the interbilayer spacing is set by the balance of van der Waals attraction of hydrocarbon slabs in water, and two repulsion forces, termed

"hydration" and "shape-fluctuation" forces, respectively.[20,24] The attractive van der Waals force can be calculated analytically for a pair of infinitely extended slabs.[25,26] The repulsive forces are described phenomenologically through empirically determined parameters and functional forms,[16,20,24]

$$F(D_W,T) = -\frac{H}{12\pi D_W^2} + P_h \lambda e^{-D_W/\lambda} + \left(\frac{k_B T}{2\pi}\right)^2 \frac{1}{K_C \sigma^2} \tag{3}$$

$$P_{osm} = -\frac{dF(D_W,T)}{dD_W} \tag{4}$$

Equation 3, an idealized form of separable terms, gives the interaction energy per membrane unit area $F(D_W = D - D_B, T)$, as a function of interlamellar water spacing, D_W, and temperature, T. Equation 4 relates the free energy, F to the applied osmotic pressure, P_{osm}. Experimental results for P_{osm} *vs.* D for DMPC in the fluid state are shown in Figure 3, together with the decomposition into the various interaction terms, adapted from reference 16.

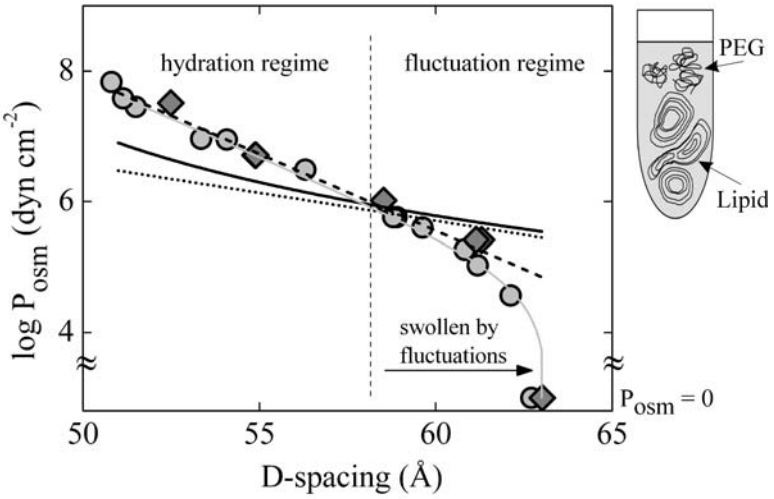


Figure 3. Variation of interlamellar repeat spacing of DMPC multilayers with applied osmotic stress. Diamonds show current data at 35°C and circles data at 30°C from Ref. 16. A hydration and a fluctuation regime are distinguished by the decomposition of forces (Equations 3-4) into van der Waals attraction (solid line), hydration repulsion (dashed), and fluctuation repulsion (dotted).

The first term in Equation 3 represents the attractive van der Waals term (solid line in Figure 3) decaying quadratically with interbilayer separation. The strength of this interaction is given by the Hamaker parameter, H, on the order of $4\text{-}5 \cdot 10^{-14}$ erg ($\approx 1\ k_B T$)

for hydrocarbon/lipids in water.[25]

The second term in Equation 3, representing the hydration force (dashed line, Figure 3), accounts for the energetic cost of water ordering in the vicinity of lipid headgroups.[27] This force is exponential, with a decay length of about 2 Å. It therefore weakens significantly as interbilayer spacings increase by 10 Å or more.

The third term in Equation 3, the shape-fluctuation or undulation term (dotted line, Figure 3) acts at larger inter-membrane separation, accounting for the entropic penalty due to confinement of undulating membranes.[28] This entropic force is inversely proportional to the bilayer bending rigidity, K_C. The value used for the plot in Figure 1 is $K_C = 0.8 \cdot 10^{-12}$ erg ($\approx 19\ k_BT$).[16] The parameter σ in Equation 3 is a function of D_W and represents the root mean-square fluctuation in interbilayer separation, $\sigma^2 = <D_W^2> - <D_W>^2$, where the brackets indicate the ensemble average over all fluctuation modes. The variation of σ with D_W required by Equation 4 can be measured experimentally by high resolution X-ray diffraction.[16,22] Note that for evaluation of forces, the natural parameter for interbilayer separation is the water spacing $D_W = D - D_B$, rather than the repeat spacing. For simplicity, the qualitative analysis presented here uses the directly measured D.

Figure 3 shows two distinct regimes. A hydration regime exists at high osmotic pressures, where the van der Waals attraction is balanced mainly by the hydration repulsion. The fluctuation force here is negligible. A second region, below 1 atm of osmotic stress, has the fluctuation force as the dominant repulsion term (fluctuation regime); here, the hydration force can be neglected. Van der Waals and hydration forces come into balance at a repeat spacing of about 58 Å (D_W = 14 Å), compared to the full free energy minimum at 63 Å (D_W= 19 Å). Fluctuations enhance swelling by 5 Å (25%).

Applying osmotic stress to sterol-containing bilayers

We can now investigate the modification of bilayer interactions by addition of sterols. In Figure 4 we plot the repeat spacings of DMPC/sterol mixtures versus sterol content. Panel A shows the full swelling at zero osmotic pressure and panel B the reduction under 0.26 atm of osmotic stress (5% PEG solutions, log P_{osm} = 5.4). There are marked differences

between the effects of sterols at full hydration (Figure 4A). For all bilayer compositions, the lamellar repeat spacing increases in the order cholesterol < lathosterol < 7-dehydrocholesterol < lanosterol. When fluctuations are suppressed by mild osmotic stress (0.26 atm), these differences are eliminated (Figure 4B).

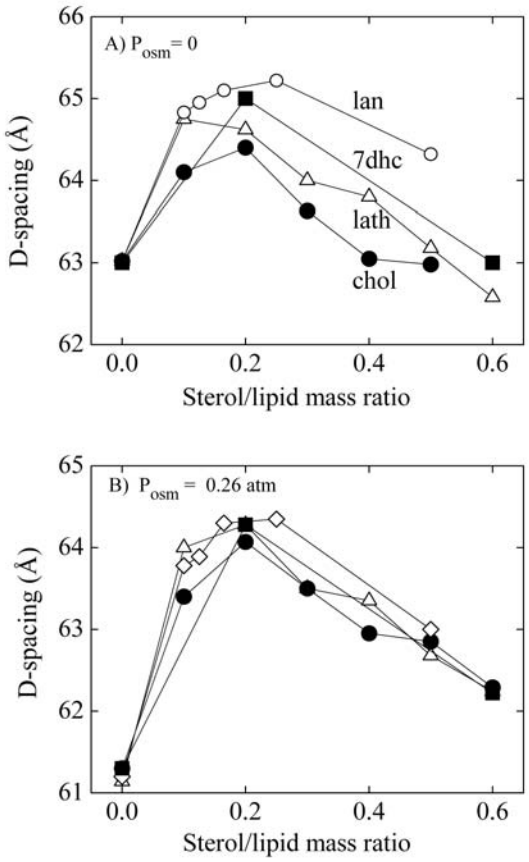


Figure 4: Interlamellar repeat spacing versus sterol content for DMPC multilayers at 35°C. (A) Large differences between sterols are seen for freely fluctuating bilayers at full hydration. (B) Differences vanish when fluctuations are suppressed by mild osmotic stress.

The modifications of interlamellar spacings by low osmotic pressure strongly suggest that sterols have noticeable effects on the bending rigidity of DMPC bilayers. In particular, cholesterol forms the most rigid bilayers (least swelling due to fluctuations), while the trimethylated precursor lanosterol makes most flexible bilayers of all sterols shown.[29-33]

The variation of *D* with sterol content in Figure 4 reflects the phase diagram of DMPC/sterol mixtures.[34-41] At 35°C, a coexistence region exists between a liquid disordered phase (with low sterol content) and a liquid ordered phase (with high sterol content). The sudden decline in the *D*-spacing values starting at a sterol/lipid mass ratio of

0.2 (30% mole sterol) corresponds to the transition into the liquid ordered phase.[34-41]

Discussion

Motivated by studies of cholesterol disorders, we have investigated the possibility that replacement of cholesterol with other sterols affects membrane bending elasticity, despite similarities in chemical structure. Indeed, seen through differences in forces of bilayer undulations, cholesterol makes significantly more rigid bilayers than its precursors, suggesting a critical role for cholesterol in modulating cellular structure and function.[42,43]

Bilayer fluctuations are suppressed by osmotic stress. For pure DMPC (Figure 3), interbilayer separation is reduced from 19 Å to about 14 Å when fluctuations are completely removed. A simple calculation using Equation 3, indicates that a similar reduction in spacing can be obtained through a 10-fold increase of the bending rigidity, K_C, when all other interaction parameters are held fixed. Measurements of K_C by various experimental methods: micropipette pressurization,[44] fluctuation microscopy,[45,46] high-resolution X-ray diffraction,[16,23] showed less than a 5-fold increase by addition of 50% cholesterol.[1,44-46] This means that bilayer fluctuations are not completely suppressed by sterols, an interpretation supported by the results in Figure 3.

We find that membrane rigidity is significantly reduced by substitution of cholesterol with other sterols, in agreement with fluctuation microscopy[46] and ^{2}H NMR[30,31] measurements. The variations with sterol type in Figure 3 can be explained by a decrease in K_C of about 15 k_BT between cholesterol and the less rigid lanosterol-containing bilayers. This difference is significant. For illustration, it would take 15 k_BT more bending energy to encapsulate a typical size protein in a liposome containing cholesterol than one with lanosterol. This energy could be accounted for by other lipid-protein interactions, for example, an additional 5 net electrostatic charges per liposome provide enough favorable binding energy for encapsulation of an oppositely charged protein.[47] This would amount to an additional 5-10% of charged lipids; in comparison, cell membranes contain about 10-20% charged lipids.

Differences between the sterol effects on membrane rigidity can be rationalized in terms of sterol location within the lipid bilayer. Cholesterol has a significant ordering effect on

the lipid acyl chains, as shown by ^{2}H NMR[1,30,40,48] and X-ray diffraction.[49-54] By contrast, only a slight effect has been detected on lipid headgroups,[55] although this may depend on lipid type and temperature[29,56]. Addition of cholesterol could create more space between lipid headgroups, thereby allowing greater headgroup disorder. It is therefore expected that sterols introduce an inhomogenous modification of lateral forces within bilayers (cf. Figure 1). The lateral stress profile and, in particular, the bending moments will therefore depend on the sterol location inside the membrane. For example, more polar sterols (due to additional double bonds) might protrude further into the headgroup/water interface than cholesterol, and render the bilayer more flexible.

We have reached a similar conclusion from measurements of DOPE/sterol mixtures forming H_{II} phases (data not shown). Cholesterol-containing DOPE forms hexagonal structures with a smaller radius of curvature than with other sterols. Quantifying changes in bending rigidity and intrinsic curvature values is in progress. Here we note that according to Equation 2, the membrane bending rigidity K_C can be modified through the monolayer bending rigidity K_C^{mono} as well as the intrinsic curvature value R_0. It is of interest to determine whether sterols affect one or both of these elastic parameters.

The behavior of interlamellar spacing with increased sterol content is not resolved by these data. From the X-ray[50,51] and ^{2}H NMR[1,31,40,48] measurements mentioned above, the bilayer thickness is estimated to increase with sterol by up to 3 Å. However, if bilayer rigidity also increases with added sterol, then a decrease in the interlamellar water spacing is expected due to weaker fluctuation repulsion. The competition between the two effects, added thickness *vs*. fluctuation suppression, could explain the peak of *D vs*. composition in part A of Figure 4. However, because the peak is still present even under osmotic stress in part B of Figure 4, it most likely reflects the variation of the bilayer thickness with increasing sterol content. It is conceivable that at 30% mole fraction (corresponding to 0.2 mass ratio) where the transition into the liquid ordered phase occurs, there is abrupt bilayer reorganization. In the liquid ordered phase, bilayer thickness might decrease with increasing sterol content, even though lipid acyl chains are stretched (as measured by ^{2}H NMR). This can be due to interpenetration of lipid chains from the two monolayers at the bilayer center to accommodate the length mismatch between the cholesterol molecule and the acyl chain of the lipids.[37,51-54,57]

The hydration repulsion can also change with the addition of sterol. This change is suggested by a picture of sterols acting as lateral spacers within the lipid matrix. A weaker hydration repulsion in the liquid ordered phase could also explain the decrease of *D* at high sterol content in Figure 4, as an alternative to a decrease in bilayer thickness discussed above. Of main interest here, however, is that modification of either hydration forces, or of bilayer thicknesses are independent of sterol type, as shown by the measurements under osmotic stress (Figure 4B). Sterol type dependence appears only in the absence of stress (Figure 4A) suggesting a stronger modification of fluctuations rather than of hydration repulsion.

As seen through modification of fluctuation forces, sterols modify bilayer bending rigidities. It is remarkable that the evolutionary process has led to cholesterol, the sterol forming the most rigid bilayers. It is therefore conceivable that, in addition to specific biochemical mechanisms, cellular manifestations of cholesterol disorders could involve modifications of membrane physical properties.

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